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Iodine Released from the Wound Dressing Iodosorb Modulates the Secretion of Cytokines by Human Macrophages Responding to Bacterial Lipopolysaccharide

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Clinical data suggests that iodine released into the wound environment by Iodosorb may enhance the healing of chronic leg ulcers by a mechanism additional to its anti-bacterial activity. The macrophage is considered to play a central role in controlling wound healing and this study was designed to determine whether interaction with iodine could modulate macrophage cytokine output. The human macrophage cell line U937 was co-cultured with Iodosorb, Iodosorb conditioned medium or elemental iodine in the presence of optimal and sub-optimal stimulatory concentrations of bacterial lipopolysaccharide (LPS). The concentration of tumour necrosis factor-α (TNFα) and interleukin-6 (IL-6) were assayed in the culture medium after 24 hr culture. Co-culture with 0.25% Iodosorb, Iodosorb conditioned medium or 20 μg/ml iodine enhanced TNFα secretion (48 ± 3% cytotoxicity in L929 bioassay to 78 ± 2% cytotoxicity, ± SD) by U937 cells stimulated with sub-optimal concentrations of LPS (0.25 ng/ml) and inhibited secretion of IL-6 from cells stimulated with 10 ng/ml LPS (> 750 pg/ml to 267 ± 52 pg/ml, ±SD, $n = 4$). Immunohistological staining of sections prepared from biopsies of chronic leg ulcers indicated that the majority of macrophages present were negative for TNFα. Thus one potential mechanism of action of iodine released from Iodosorb used as a wound dressing is to provide a pro-inflammatory stimulus in the wound tissue by activation of the resident macrophage population. This would result in a localized production of pro-inflammatory cytokines and generate an influx of monocytes and T-lymphocytes into the wound that may trigger the wound into a healing phase.

Keywords: Interleukin-6 Iodine Macrophage Tumour necrosis factor-α Wound healing

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INTRODUCTION

Normal wound healing is a complex ordered process involving cell–cell communication via soluble molecules such as chemokines, cytokines and growth factors interacting in a dynamic network (Blistein-Willinger, 1991) to achieve efficient wound closure. By inference a contributory factor to defective healing in chronic lesions such as leg ulcers, decubitus ulcers and diabetic ulcers may be a disordered regulatory network. Restoration of the appropriate balance of these factors would then lead to an appropriate therapeutic approach to initiate healing of the chronic lesion.

One key cellular component involved in healing is the generation of an inflammatory response resulting in the infiltration of the wound margin and wound bed by mononuclear leukocytes, particularly macrophages (Dyson *et al.*, 1988). The presence of these cells at an inflammatory site is dependent upon cytokine generation (Lukacs *et al.*, 1995) and they are themselves a rich source of cytokines (Nathan, 1987) regulating the healing process. Enhancement of wound healing by application of exogenous cytokines or growth factors is an

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attractive concept under current investigation (Brown *et al.*, 1994) and cellular mechanisms can be identified to support this approach (Cromack *et al.*, 1990). An alternative approach is to devise a therapy, which regulates cell function so that cells present at the wound site may be manipulated to modulate their cytokine production *in situ*. The responsiveness of macrophages to localized stimuli by modifying their cytokine output (Heumann *et al.*, 1994) renders them an ideal target for such immunotherapy.

Iodosorb is a wound dressing material comprising particles manufactured from cross-linked polymerized dextran containing 0.9% w/v iodine. As the Cadexomer hydrates in the moist wound environment elemental iodine is released to exert an antibacterial effect and possibly interact with cells within the wound. Iodosorb has been shown to accelerate the rate of epithelization of venous ulcers when compared to standard treatments in a number of studies (Skog *et al.*, 1983; Harcup and Saul, 1986; Ormiston *et al.*, 1985). Whilst treatment with iodine containing dressings is primarily intended as an anti-bacterial treatment clinical evidence suggests that Iodosorb treatment may exert a healing stimulus, which is independent of the elimination of infection (Skog *et al.*, 1983; Kero *et al.*, 1987).

Iodine is bioactive in that it has been shown to be an essential co-factor in neutrophil (Clark and Klebanoff, 1975) and monocyte/macrophage (Nathan *et al.*, 1979) cytocidal activity generated via a myeloperoxidase/hydrogen peroxide pathway initiated as a consequence of phagocytosis. Normal immunologic function can be radically affected by sub-toxic concentrations of povidone iodine equivalent to 10^{-4} M iodine. Thus *in vitro* antigen driven (mixed lymphocyte culture) and mitogenic (phytohaemagglutin) responses are down regulated by the generation of suppressor cells (Ninneman and Stein, 1981). These effects are similar to those demonstrated by direct periodination of lymphocytes where the generation of suppressor activity was induced as a consequence of modifications of membrane sugar residues (Galanoud *et al.*, 1980).

We initiated this study therefore to determine whether iodine released into the wound environment by Iodosorb may act to modify the healing process by interacting with macrophages to modulate cytokine production and thus indirectly influence the healing of chronic wounds.

MATERIALS AND METHODS

Cell lines

The human histiocytic lymphoma cell line U937 and the murine fibroblast cell line L929 were maintained in continuous culture in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), penicillin, streptomycin and fungizone (RPMI/10%).

Preparation of U937 for in vitro *activation assays*

Proliferating U937 cells were suspended at 2×10^5/ml in RPMI/10% containing 2.5×10^{-7} M phorbol myristate acetate (PMA) and 1 ml was added to each well in a 24-well plastic tissue culture plate. Each plate was incubated at 37°C for 48 hr to allow differentiation of the proliferating non-adherent parent cell line to adherent cells with a macrophage like phenotype (DU937). Preliminary experiments indicated the PMA concentration and time used were optimal to produce the maximum number of adherent cells.

Preparation of Iodosorb, Cadexomer and iodine for in vitro *use*

Aliquots of the Cadexomer supplied (Batch Number 930923) and Iodosorb (Batch Number 940418) (Perstorp Pharma, Sweden) were incubated at 37°C in tissue culture medium containing no antibiotics and demonstrated to be sterile. Both were thereafter stored in sterile plastic universal containers. For use small amounts were dispensed into pre-weighed plastic bijou containers, re-weighed and the appropriate amount of RPMI containing 15% FCS (RPMI/15%) to yield the desired w/v concentration was added immediately before addition to the DU937 monolayers.

Stock iodine solution was prepared as a 1% w/v solution in aqueous 2% potassium iodide and diluted in RPMI/15% for use.

Toxicity was determined by co-culture of DU937 monolayers with Cadexomer or Iodosorb for 24 hr. The Cadexomer or Iodosorb was removed by gently washing with Dulbecco's salt solution (DSS) and the number of cells remaining on the monolayer evaluated microscopically. Viability of remaining adherent cells

was determined by microscopic evaluation of Trypan Blue exclusion (viable cells exclude Trypan Blue, dead cells are stained blue).

U937 activation experiments

Prior to addition of stimulatory agents [lipopolysaccharide (LPS) prepared from *Escherichia coli* (Sigma, U.K.), Cadexomer, Iodosorb, Iodosorb conditioned medium or iodine] to DU937 monolayers non-adherent cells were gently resuspended by washing the monolayers with tissue culture medium and the medium in each well replaced with 1 ml of RPMI/15% and stimulating agent as appropriate.

DU937 monolayers were incubated with stimulating agents for 24 hr in order to allow cytokine production. Supernatants were centrifuged at 13 000 rpm for 5 min to remove any contaminating DU937 cells and the cell free supernatants were stored at −20°C for subsequent analysis.

Tumour necrosis factor-α bioassay

L929 cells were plated out in RPMI/10% in 75 ml aliquots each containing 4.5×10^3 cells in 96-well tissue culture plates and incubated for 20 hr at 37°C. A 75 μl portion of test supernatant diluted 1:50 was added in quadruplicate to wells containing L929 cells.

A negative control of medium alone and positive controls containing 2.5, 5.0 and 10 pg/ml of recombinant tumour necrosis factor-α (TNFα) to ensure that the assay cells were sensitive to the action of TNFα were also added to L929 monolayers. Each well was supplemented with actinomycin D at a working concentration of 2 μg/ml and the plates incubated for a further 18 hr. The supernatant from each well was discarded, each well washed once with DSS and 100 μl of 0.5% aqueous crystal violet added to each well for 20 min. Each well was washed with tap water × 10 and the plates air dried, then 100 μl of 0.1% acetic acid was added to each well and the stain eluted from the cell monolayer with gentle shaking. The OD_{540} for each well was measured using a microplate reader, the mean for each test situation calculated and the blank value for wells incubated with medium alone (no cells present) subtracted from each test value.

Calculation of TNFα activity

TNFα activity was calculated as follows:

$$\% \text{ Cytotoxicity} = \frac{OD_{540}\ \text{Control} - OD_{540}\ \text{Test}}{OD_{540}\ \text{Control}} \times 100$$

where

control value = OD_{540} of cells incubated in medium alone − blank

and

Test value = OD_{540} of cells incubated with TNFα containing medium

This assay does not provide absolute concentrations of TNFα. However, increased % cytotoxicity correlates with increased concentration of TNFα. In comparisons of test to control (e.g. assays of supernatants from DU937 cells cultured in the presence or absence of an appropriate stimulus) a higher % cytotoxicity in the test indicates enhancement of TNFα secretion.

ELISA assays

Twin site sandwich ELISA based assay kits for human TNFα or interleukin-6 (IL-6) were obtained from CLB, Netherlands and U937 culture supernatants assayed directly. The absolute cytokine concentration was determined by use of a calibration curve prepared using standards provided by the kit manufacturer. The culture medium did not contain detectable amounts of either TNFα or IL-6.

Immunohistology

Following study approval by the Local Research Ethics Committee and after obtaining informed patient consent 6 mm punch biopsies were taken under local anaesthesia from the bed of chronic leg ulcers. These ulcers had been present for a minimum of 6 months and clinical records showed no evidence of healing occurring in the 6 weeks prior to biopsy.

Biopsies were snap frozen in liquid nitrogen and 6 μm cryostat sections mounted on poly-L-lysine treated microscope slides. Slides were stored desiccated at −20°C for up to 14 days prior to staining. Sections were fixed in dry acetone, washed in phosphate buffered saline (PBS) three times and incubated for 30 min in optimal dilutions of either anti-TNFα monoclonal antibody (MAB) or MAB specific for the macrophage associated CD68 antigen. They were then washed three times in PBS and antibody localization identified by a standard streptavidin-biotin peroxidase tec-

nique (Vector Laboratories, Peterborough, U.K.) with final reaction product developed using 3,3′-di-aminobenzidine (DAB). The sections were counterstained with Ehrlich's haematoxylin, dehydrated, cleared and mounted in DPX mounting medium. Positive staining was seen as a brown–black deposit and non-stained cells could be clearly distinguished as blue counterstained nucleated cells with no associated brown DAB stain.

The anti-TNFα MAB was prepared from the hybridoma cell line 2-179-E11 (Meager *et al.*, 1987) obtained from the European Collection of Cell Cultures and the anti-CD68 MAB purchased from Dako Ltd, High Wycombe, U.K.

RESULTS

Toxicity of iodine released from Iodosorb for DU937 cells was evaluated over a concentration range up to 2% w/v Iodosorb in two experiments. Toxicity at concentrations above 0.5% w/v Iodosorb were manifested as a decrease in adherent cell number and 0% viability as determined by Trypan Blue exclusion of the few remaining adherent cells. Cadexomer proved less toxic with only slight toxicity being identified at concentrations as high as 2% w/v. Culture medium treated with 0.5% Iodosorb overnight and then assayed in the L929 cytotoxicity assay demonstrated no spurious cytotoxic effect.

It would appear therefore that the toxicity of Iodosorb is a consequence of the iodine constituent when present in excess of an equivalent of 0.0045% elemental iodine (3.5×10^{-3} M). 0.5% Iodosorb or Cadexomer as control was therefore selected as the upper limit for dose-response evaluations in DU937 activation experiments.

Effect of Iodosorb and iodine on production of TNFα by DU937 cells

Initial experiments to determine whether Iodosorb enhanced TNFα production were performed using medium supplemented with 15% serum containing > 100 EU/ml of LPS assayed by the manufacturer. These experiments indicated that 0.25% w/v Iodosorb induced a maximal threefold increase of secreted TNFα over that in the presence of medium alone. Thus DU937 cultured in medium alone for 24 hr produced 0.4 pg/ml of TNFα whilst culture in the presence of 0.25% Iodosorb induced production of 1.2 pg/ml TNFα. Concentrations > 0.25%, which approached the toxic concentration of Iodosorb had no effect on TNFα secretion. The use of an immunologically specific ELISA demonstrated that Iodosorb did induce production of TNFα. All subsequent assays for TNFα were performed using the L929 cytotoxicity assay.

Iodosorb is yellow in colour and it rapidly decolourizes upon addition to culture medium indicating that iodine is lost to the medium. Control experiments indicated that Cadexomer alone had no effect on TNFα production (Table 1). To demonstrate that enhanced secretion of TNFα was mediated by the iodine released from Iodosorb DU937 cells were incubated either directly with 0.25% Iodosorb or with medium, which had been pre-incubated with Iodosorb and the Cadexomer carrier removed by centrifugation (Table 2). Whilst these experiments indicated that enhanced TNFα secretion was a consequence of release of iodine into the medium by Iodosorb it was not possible to determine whether the iodine mediated the effect alone or whether DU937 cells required co-stimulation with LPS.

Table 1. Cadexomer alone does not induce TNFα secretion by DU937 cells

Culture conditions:		
Medium only	0.25% Cadexomer in culture medium*	0.25% Cadexomer in conditioned medium†
% Cytotoxicity TNFα assay		
17 ± 4	13 ± 4	10 ± 1

*Cadexomer added directly to DU937 monolayer cultures for 24 hr prior to supernatant harvest for TNFα assay.

†Cadexomer incubated with RPMI/15% for 24 hr at 37°C, Iodosorb removed by centrifugation and DU937 monolayers cultured in the supernatant medium for 24 hr prior to supernatant harvest for TNFα assay.

Cultures performed in medium supplemented with 15% FCS containing > 100 EU/ml; each result is the mean of four replicate cultures ± SD.

Table 2. Iodosorb or Iodosorb conditioned medium enhances TNFα secretion by DU937 cells

Experiment No.	Culture conditions:		
	Medium only	0.25% Iodosorb in culture medium*	0.25% Iodosorb conditioned medium†
	% Cytotoxicity TNFα assay		
1	27 ± 7	62 ± 11	58 ± 16
2	27 ± 3	37 ± 19	43 ± 10
3	28 ± 11	51 ± 4	62 ± 11

*Iodosorb added directly to DU937 monolayer cultures for 24 hr prior to supernatant harvest for TNFα assay.
†Iodosorb incubated with RPMI/15% for 24 hr at 37°C, Iodosorb removed by centrifugation and DU937 monolayers cultured in the supernatant medium for 24 hr prior to supernatant harvest for TNFα assay.
Cultures performed in medium supplemented with 15% FCS containing > 100 EU/ml; each result is the mean of four replicate cultures ± SD.

Culture of DU937 cells in medium supplemented with 15% FCS containing < 10 EU LPS/ml (assayed by manufacturer) did not stimulate significant levels of TNFα production (< 10% cytotoxicity) and co-culture with 0.25% Iodosorb did not enhance this. As the concentration of LPS was increased over the endogenous levels TNFα secretion increased until maximal secretion was achieved at 1 ng LPS/ml. Co-culture with Iodosorb enhanced TNFα secretion at suboptimal levels of LPS. At 0.25 pg/ml LPS, which induced a 50% of maximal response co-culture with 0.25% Iodosorb enhanced TNFα output as demonstrated by an increase from 48 ± 3% cytotoxicity to 78 ± 2% cytotoxicity (± SD, $n = 4$) in the TNFα bioassay (Fig. 1). Slight enhancement of TNFα secretion was exhibited when 0.25% Iodosorb was added to DU937 cells cultured with 0.5 and 0.75 pg/ml LPS. Essentially identical results were obtained when the experiment was repeated substituting 20 μg/ml iodine dissolved

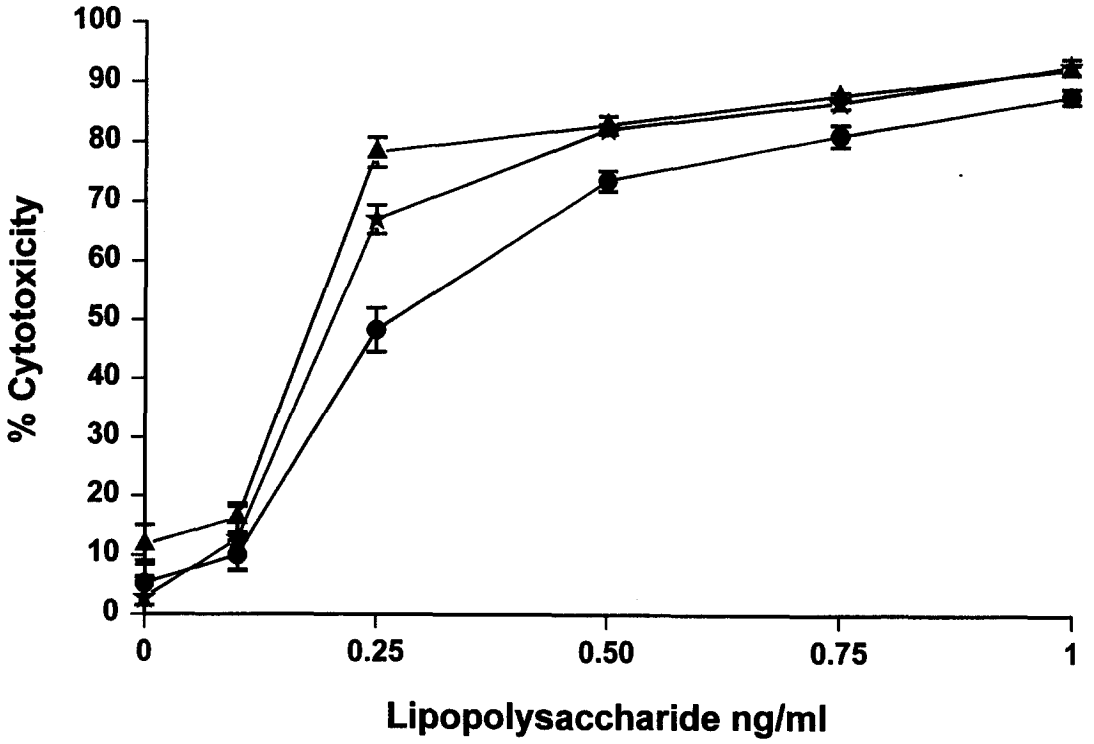

Fig. 1. Iodosorb or iodine synergises with a suboptimal concentration of LPS to enhance TNFα production by DU937 cells. (▲) 0.25% Iodosorb, (★) 20 μg/ml iodine, (●) LPS alone. Each result is the mean of four replicate DU937 cultures. $P < 0.001$ (Student's t-test) for LPS alone vs cultures containing LPS + Iodosorb or LPS + iodine at LPS concentrations of 0.25, 0.5 and 0.75 ng/ml.

Table 3. Iodosorb and Iodosorb conditioned medium suppresses LPS induces IL-6 secretion by DU937 cells

DU/937 culture conditions	IL-6 (pg/ml)
1. Control—no LPS/no Iodosorb	24 ± 1
2. 10 ng/ml LPS	> 750
3. 10 ng/ml LPS + 0.25% Iodosorb*	267 ± 52
4. 0.25% Iodosorb	19 ± 6
5. 0.01 mg/ml LPS + medium preincubated with 0.25% Iodosorb†	275 ± 54

*Iodosorb added directly to DU937 monolayer cultures for 24 hr prior to supernatant harvest for IL-6 assay.

†Iodosorb incubated with RPMI/15% for 24 hr at 37°C, Iodosorb removed by centrifugation and DU937 monolayers cultured in the supernatant medium for 24 hr prior to supernatant harvest for IL-6 assay.

Each result is the mean of four replicate cultures ± SD.

in potassium iodide solution for Iodosorb (Fig. 1).

Effect of Iodosorb on DU937 production of IL-6 by DU937 cells

To determine whether the stimulation of TNFα secretion by Iodosorb/iodine was a manifestation of a generalized stimulation of DU937 cytokine secretion the concentration of IL-6 was monitored in culture supernatants where enhanced levels of TNFα had been demonstrated after Iodosorb co-culture. LPS enhanced IL-6 secretion but no additive effect could be demonstrated in the presence of Iodosorb. After maximal stimulation of DU937 with 10 ng/ml LPS co-culture with 0.25% Iodosorb or Iodosorb-conditioned medium was found to reduce production of IL-6 by > 60% but did not induce complete inhibition (Table 3).

Distribution of TNFα positive cells in chronic wound tissue

Granulation tissue taken from the wound bed of chronic leg ulcers contains large numbers of macrophages, which may be identified by staining with MABs specific for CD68 antigen [Fig. 2(top panel)]. In biopsies taken from non-infected wounds the majority of these macrophages do not stain with a MAB specific for TNFα [Fig. 2(bottom panel)]. Cytoplasmic staining of TNFα can however be demonstrated in cells associated with some but not all capillaries within the granulation tissue (Fig. 3).

DISCUSSION

Iodosorb is used clinically to treat infected wounds by absorption of wound exudate and release of iodine at the wound surface. Data exists to indicate that Iodosorb may enhance the healing of chronic leg ulcers by a mechanism additional to its anti-bacterial effect. For example in one clinical trial significant wound re-epithelialization was demonstrated in the Iodosorb treated group even though only 42%

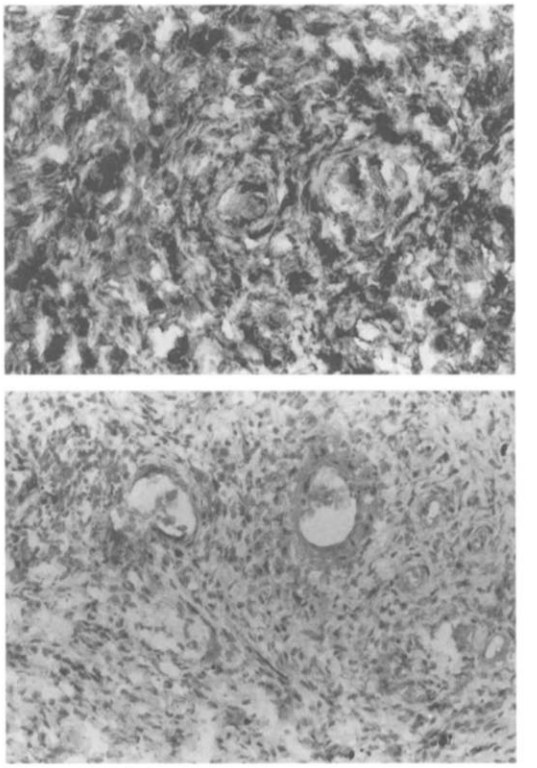

Fig. 2. Macrophages within the wound bed of chronic leg ulcers do not stain for TNFα. (Top) Cryostat section of punch biopsy taken from a non-healing chronic leg ulcer and stained to identify $CD68^+$ macrophages. Positive cells are identified by black staining of the whole cell area. In this area of the section > 50% of the cells present are positive. Original magnification × 104. (Bottom) Cryostat section of the same biopsy as shown in the top panel stained to identify TNFα expressing cells. The area shown is approximately the same as in the top panel. Original majority of cells are negative for TNFα with only haematoxylin counterstained nucleii showing staining. Only isolated positive cells can be identified close to one blood vessel. Original magnification × 104.

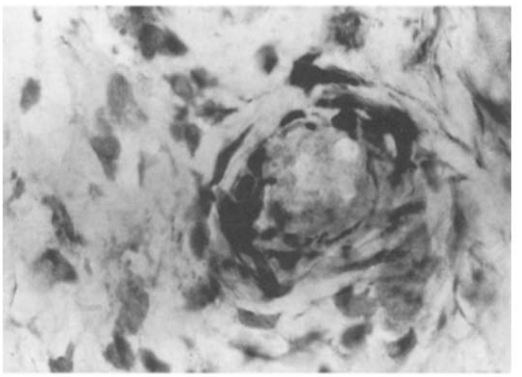

Fig. 3. TNFα positive cells identified in perivascular area of wound bed tissue from a chronic non-healing leg ulcer. Cryostat section of the same biopsy as shown in Fig. 2 stained to identify TNFα expressing cells. An area of the section was selected that demonstrated perivascular cells staining strongly for TNFα. Positive cells can be seen as black stained cells in a close perivascular orientation. Cells distant to the vessel are negative and only exhibit the haematoxylin nuclear counterstain. Original magnification × 520.

(16/38) of the wounds were demonstrated to have had an infection eliminated (Skog *et al.*, 1983). The objective of this project was therefore to determine whether Iodosorb and its active component iodine may act to enhance wound healing by modification of cellular mechanisms important in regulating the healing process.

Macrophages are a rich source of cytokines and growth factors that may be instrumental in regulating healing (Nathan, 1987). Paradoxically chronic wound tissue is heavily infiltrated with macrophages and to a lesser extent T-lymphocytes. The cytokine profile of macrophages is modulated upon activation and the possibility exists that chronic wound macrophages are either not functionally activated or are inappropriately activated. We have previously demonstrated that within chronic wounds only recently extravasated macrophages close to microvessels at the wound margins exhibit membrane activation markers (Moore *et al.*, submitted for publication). In the majority of chronic leg ulcers the macrophages distant to vessels are non-activated as demonstrated by non-expression of the receptor for the third complement component, C3b, and for the immunoglobulin FcγIII receptor. A possible mechanism of action of iodine may be to induce macrophage activation within the chronic wound and thus modulate macrophage cytokine output.

We selected the human histiocytic lymphoma cell line U937 as a target cell in our study as it has been widely used as a model for the human macrophage. Our data clearly demonstrate that Iodosorb can modulate the secretion of cytokines by this cell line. Co-culture of adherent U937 cells with 0.25% Iodosorb enhances TNFα production in the presence of LPS at concentrations below that which induces maximal stimulation. The same concentration of Iodosorb also suppressed IL-6 secretion after stimulation with an optimal concentration of LPS. IL-6 has been demonstrated to down-regulate the production of TNFα by U937 cells and human monocytes in response to LPS *in vitro* and also at the whole organism level in mice (Aderka *et al.*, 1989). This may represent the negative arm of a regulatory circuit controlling the response of macrophages to inflammatory stimuli. By providing either a direct stimulus for TNFα secretion or by inhibiting negative regulation iodine may act as a pro-inflammatory stimulus within the wound environment.

As Iodosorb hydrates in a moist wound environment elemental iodine is released to exert an antibacterial effect and possibly interact with cells within the wound. A similar effect is observed *in vitro* and iodine is lost to the culture medium. Our data clearly demonstrates that the effect on macrophage cytokine production is mediated by iodine as co-culture of DU937 cells with either Iodosorb, Iodosorb conditioned medium or elemental iodine mediates the same effect. Of particular relevance is that the effect on TNFα production is only manifest at concentrations of LPS sub-optimal for DU937 activation. A chronic wound, which is not clinically infected is still likely to be colonized with low levels of skin bacteria resulting in the presence of low levels of LPS in the wound tissue that would be sub-optimal for macrophage activation. In the absence of an appropriate stimulus from the cytokine network macrophages will remain non-activated. This concept is supported by our demonstration that the majority of macrophages in chronic wound tissue are negative for TNFα with positive cells only identified close to blood vessels. Thus interaction with iodine in the wound environment may induce activation of wound macrophages and release of *inter alia* TNFα with this effect being amplified by a concomitant down regulation of IL-6 production.

Release of TNFα by wound macrophages as a means of enhancing wound healing is possibly a controversial point. However, topical application of TNFα in a rat wound model has been

demonstrated to enhance healing (Schlenger *et al.*, 1994) whilst other workers have produced data to suggest that TNFα inhibits granulation tissue formation in a different rat model (Rapala *et al.*, 1991). An inflammatory response is known to be required for normal healing and despite the presence of inflammatory cells within chronic wounds healing does not progress. The generation of an inflammatory response within chronic wound tissue may re-initiate the healing process so that the normal sequence of events are triggered in the chronic wound environment. TNFα would be an ideal candidate molecule for this purpose and intradermal injection of TNFα to human volunteers has been demonstrated to generate an infiltrate of macrophages and T helper cells and also induce expression of endothelial cell and keratinocyte adhesion molecules (Groves *et al.*, 1995).

Macrophages within wound tissue may provide an ideal target for stimulation by appropriate molecules delivered to the wound environment. We have demonstrated in this paper that iodine enhances macrophage TNFα secretion *in vitro* and that the majority of macrophages within chronic non-infected wound tissue produce little detectable TNFα. The possibility therefore exists that delivery of iodine to non-activated macrophages within the chronic wound may induce TNFα as a primary event and as a consequence induce a fresh influx of macrophages and T helper cells, which are considered to play a positive role in modulating wound healing (Barbul *et al.*, 1989). Testing of this hypothesis awaits the outcome of experiments to evaluate wound macrophage cytokine secretion after application of Iodosorb directly to chronic wound tissue.

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